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van Zutphen, Tim; van der Klei, Ida J.

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Quantitative analysis of organelle abundance, morphology and dynamics

Tim van Zutphen and Ida J van der Klei

Recent data indicate that morphological characteristics of cell organelles are important for their function in the cell. These characteristics include not only their shape, number and size, but also their distribution in the cell. Moreover, the dynamics of processes that result in changes in these characteristics (e.g. organelle fission, fusion, autophagy, transport) influence the function of the cell.
For a better understanding of these processes quantitative approaches are important. Here we give an overview of contemporary biochemical and microscopy methods that are used to quantify organelle abundance, morphology and the kinetics of the processes that cause changes in these properties.

Address
Molecular Cell Biology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), Kluyver Centre for Genomics of Industrial Fermentation, University of Groningen, Haren, The Netherlands

Corresponding author: van der Klei, Ida J (I.J.van.der.klei@rug.nl)

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Introduction

In systems biology computational models of biological processes are generated based on quantitative data. At present most data are generated by omics approaches that measure the quantities of a large variety of biomolecules (e.g. RNA, proteins, metabolites). A complication in systems biology of eukaryotic cells is the presence of subcompartments, the organelles. Therefore, not only the average cellular concentration of certain molecules, but also their distribution over the subcompartments is important. Moreover, in addition to the volume fraction and chemical composition of cell organelles, their morphology and dynamics are relevant for understanding cellular processes as well.

A striking example of the importance of organelle morphology in cellular function is the observation that enhancing peroxisome numbers by overproduction of a peroxisomal membrane protein results in a significant increase in penicillin production in *Penicillium chrysogenum* [1]. In this example the levels of peroxisomal enzymes involved in penicillin biosynthesis were unchanged. In the control strain these enzymes were packed in a few large organelles, whereas in the engineered strain these enzymes were distributed over a larger number of smaller organelles, which apparently resulted in higher penicillin biosynthesis.

Another example is the effect of a change in mitochondrial morphology on malate production in *Saccharomyces cerevisiae*. Mitochondria continuously divide and fuse. Upon deletion of *FIS1* mitochondrial fission is blocked, but fusion continues resulting in the formation of an interconnected mitochondrial network. Interestingly, in *fis1* cells malate production is enhanced during sake brewing [2].

Using biomimetic networks composed of micrometer-sized compartments joined together by nanotubes, Lizana *et al.* showed that shape changes indeed affect the activities of the enzymes that were enclosed in these compartments [3].

Hence, correct understanding of processes in eukaryotic cells requires a detailed description of organelle abundance and morphology, as well as of the kinetics of the processes that cause fluctuations in these parameters (e.g. organelle fission, fusion, autophagy, segregation, Figure 1).

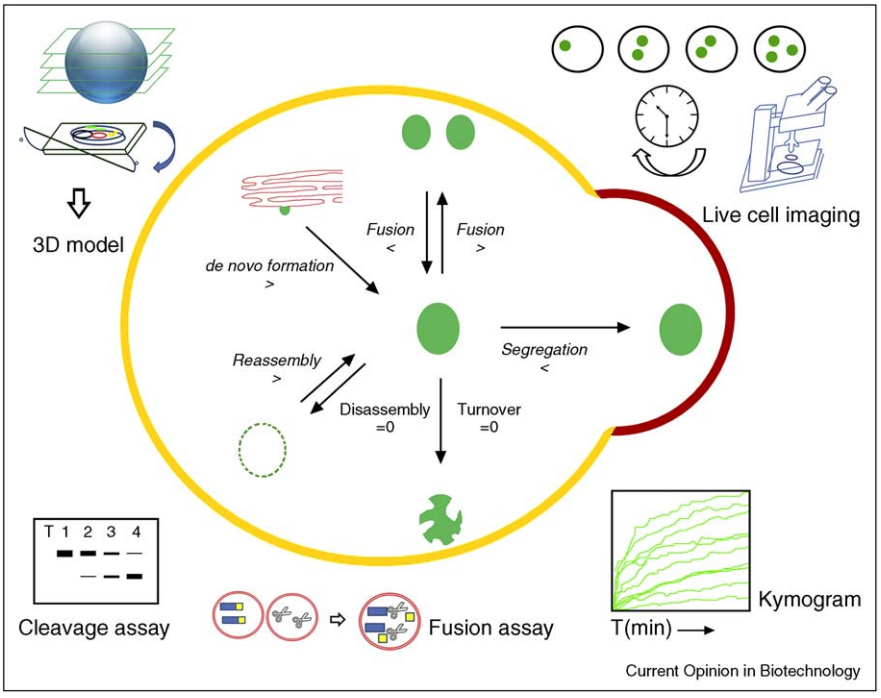
In this review we discuss approaches to measure these parameters by biochemical and microscopy techniques.

Quantifying organelle abundance and morphology

Electron microscopy and soft X-ray imaging

For the analysis of organelle abundance and morphology electron microscopy (EM) is the method of choice because of the high resolution (approx. 2 nm). Also, EM has the advantage that all organelles are visualised and not only those that are fluorescently labelled, as in fluorescence microscopy (FM). However, EM has also disadvantages such as the risk of introducing morphological changes by fixation and embedding and the fact that living cells (and hence organelle dynamics) cannot be analysed.

Figure 1



Schematic representation of organelle homeostasis in eukaryotic cells. Several organelles like mitochondria, vacuoles and peroxisomes (indicated as green structures) can be formed by fission of pre-existing ones, leading to an increase in organelle number (indicated by >). The opposite, organelle fusion, can also occur for mitochondria and vacuoles, leading to a decrease in organelle number (indicated by <). Peroxisomes, vacuoles and the Golgi apparatus can also be formed *de novo* (>), for instance from the endoplasmic reticulum (indicated in red). Entire organelles can be removed from the cell by autophagy (=0). During cell division organelles are divided over the mother and daughter cells leading to a decrease in organelle number in the mother cell (indicated by the yellow cell membrane) and an increase in organelle number in the daughter cell (indicated by red cell membrane). Some organelles (e.g. the nucleus in mammalian cells) can disassemble and reassemble. To measure organelle numbers and dynamics several techniques are available (presented outside the cell). These include live cell imaging (upper right corner), together with the generation of kymograms from the movies (lower right corner). Using confocal laser microscopy or by the analysis of serial sections by electron microscopy, 3D models can be made from cells (upper left corner). Based on these models accurate data on organelle number and morphology can be obtained. Biochemical approaches exist to study fusion or degradation of organelles. These are generally based on cleavage of a reporter protein (lower left corner), leading to a cleavage product that is visualised as a low molecular weight band by Western blot analysis or activation of a protein upon cleavage (see text for details on the depicted techniques).

EM is effectively used to accurately calculate organelle volume fractions. Also, it has been proven to be suitable to measure autophagy, by quantifying the cytoplasmic area occupied by autophagosomes [4,5].

EM is less suitable to measure the total number of organelles per cell or the 3D morphology of organelles, unless 3D reconstitutions are made based on serial sections or tomography. The latter techniques however are very time consuming and not appropriate for the analysis of large numbers of cells.

A relatively new method is soft X-ray imaging, which is a fast imaging technique that allows quantitative analysis of organelles up to 20 nm resolution [6•]. Like in EM there is no need for introducing fluorescent marker proteins or dyes. However, the availability of X-ray tomography facilities is still limited and requires the availability of a synchrotron.

Fluorescence microscopy (FM)

FM (wide field and confocal laser scanning microscopy, CLSM) is most commonly used for quantitative measurements of cell organelles, however, the resolution is relatively low (up to 200 nm) compared to electron microscopy. With the introduction of novel FM techniques, which have a much higher resolution (e.g. PALM, STED; recently reviewed [7]), FM based quantitative data are expected to become more accurate in the near future.

The simplest way to describe organelle abundance is counting fluorescently labelled structures manually/visually; the most advanced method is using an automated high throughput (HT) and high-content imaging (HC) approach, which involves automated image acquisition and quantification of a large number of different morphological parameters using image processing software.

For instance, the entire collection of viable *S. cerevisiae* deletion strains, producing the peroxisomal matrix marker GFP-PTS1, was automatically analysed for the presence of peroxisomes using high-resolution, multidimensional confocal imaging and a software tool designed for automated processing and quantitative analysis of acquired multichannel three dimensional image data [8]. This study resulted in the identification of all previously known proteins required for import of peroxisomal matrix proteins that contain a peroxisomal targeting signal 1 (PTS1), thereby validating the approach. In addition, two novel proteins involved in import of PTS1 proteins were identified, illustrating the power of this technique in screening procedures.

Many advanced automated image analysis methods are currently available, including commercial software tools as well as open source tools (for reviews [9,10]). Because of the high diversity in research topics and model organisms, no single software fits all needs and adaptations of the software are generally required [10••]).

Typical elements of automated image analysis include segmentation (identification of the cells), classification (selection of the cells to be measured), measurement of a specific property (morphological features) and quantitative analysis of the data [9].

Although for spherical organelles the quantification of numbers eventually combined with their diameters may give sufficient information, generally more complex information on organelle abundance and morphology is required.

For instance, mitochondria can show a great variation in morphology, ranging from spherical organelles to elongated or branched ones. Koopman *et al.* [11] developed an elegant method to describe the morphology of each mitochondrion using two parameters: the aspect ratio (AR) and the form factor (F). The AR is the ratio between the major and minor axes of organelle and F the perimeter$^2/4\pi\cdot$area. Both F and AR have a minimal value of 1 (corresponding to a perfect circle). Plotting of AR as a function of F revealed that AR is a measure of mitochondrial length, whereas F is a measure of both length and degree of branching [12]). These plots therefore give a detailed quantitative description of mitochondrial morphology.

An example of an advanced image processing programme for yeast is CalMorph [13], which allows to evaluate 1111 quantitative parameters to describe the morphology of a yeast cell and most of its subcellular structures. These include properties such as the morphology of the cell (size, length, shape) as well as organelle numbers, shape, size, orientation, location relative to other structures, and so on.

An impressive example of HT-HC microscopy was recently reported by Vizeacoumar *et al.* [14••], who used HT-HC microscopy to examine fluorescently labelled mitotic spindles. In this study the spindle orientation relative to the cell axis in budding yeast cells was analysed in wild-type cells and thousands of single and double deletion mutants.

For each strain 2D histograms were prepared that describe the orientation of the spindle based on the analysis of FM images of 35,000 cells. In these histograms the *X*-axis represents the budding index (i.e. the ratio of daughter to mother area) and the *Y*-axis the orientation of the spindle relative to the mother-bud axis (ranging from 0, which is parallel to the mother-bud axis, to 90°, which is perpendicular to the mother-bud axis). Upon comparing histograms from wild-type and mutant strains several novel genes that potentially play a role in spindle orientation were identified [14••].

Measuring organelle dynamics

The steady state numbers of organelles in a cell are the result of processes that increase (e.g. fission) and reduce (e.g. autophagy, segregation) organelle numbers, whereas the distribution of organelles is the result of intracellular transport and retention processes.

An *in vitro* biochemical assay is available to measure organelle fusion. Biochemical assays are also available to monitor autophagy *in vivo*. For all other processes only microscopy based methods are available. To measure the dynamics of processes in individual cells, the microscopy methods are limited to those that can be used without fixation (i.e. live cell imaging). Therefore electron microscopy and immunofluorescence are excluded.

To follow dynamic processes in time, many images are made from the same cell in live cell imaging. This can be troublesome when the fluorophore is rapidly bleached or when exposure of the cells to the excitation light results in phototoxicity.

Biochemical assays

To measure the kinetics of organelle fusion *in vitro* assays have been established [15,16], which are based on mixing of organellar contents of different composition after *in vitro* fusion. Only upon mixing of the contents a biochemical process occurs that can be monitored. The organelles with different protein contents are isolated from two different strains. For instance, in one strain the organelle contains an inactive pro-enzyme, whereas in the other strain the organelle does not harbour this pro-enzyme, but contains a protease that cleaves and activates the pro-enzyme. When these organelles are isolated and mixed, *in vitro* fusion results in activation of the pro-enzyme, which can be measured by an enzyme assay [16].

Autophagic degradation of an organelle can be measured biochemically by *in vivo* pulse-chase experiments, in which the fate of radiolabelled organellar proteins is followed in time. In addition, Western blot based quantitative biochemical approaches exist to measure autophagy *in vivo* [17]. The most used one in yeast relies on the cleavage of Atg8-GFP in the vacuole. Atg8-GFP is localised to autophagosomal membranes and a portion of this protein is trapped inside mature autophagosomes during macroautophagy [18]. Upon fusion with the vacuole Atg8-GFP is cleaved by vacuolar proteases resulting in the formation of free GFP, which can be visualised as an extra protein band by Western blotting using anti-GFP antibodies. A similar approach has been used to measure the kinetics of autophagic degradation of peroxisomes, using a strain producing a peroxisomal membrane protein fused to GFP-fusion (Pex14-GFP; [17]). This approach can be used to monitor autophagic degradation of any organelle that is tagged with a GFP fusion protein.

Microscopy assays

Organelle movement

Pepperkok *et al.* [19] was one of the first who presented an example of a method to determine the kinetics of vesicular transport in mammalian cells. In this example, vesicle transport from the ER via the Golgi apparatus to the plasmamembrane was measured. A GFP fusion of a temperature sensitive variant of a plasmamembrane protein accumulated at the ER at the constrictive temperature and, upon shifting to the permissive temperature, was transported to the plasma membrane. Cells were fixed at different time points after the temperature shift and analysed by immunofluorescence, followed by automated image acquisition and analysis, resulting in ratios of fluorescence intensities at the plasma membrane and inside the cell. These ratios could be used to calculate transport rates [19]. The same method should in principle be applicable to monitor the formation, transport and fusion of other transport vesicles in eukaryotic cells.

The most commonly used method to study the movement of fluorescently labelled organelles is live cell imaging. The data can be used to prepare a kymogram, which gives a graphical representation of the spatial position of the fluorescent structures over time and facilitates the measurement of the rate of movement of organelles. This method is however also suitable to monitor and quantify organellar fission and fusion events.

Fluorescence correlation spectroscopy (FCS) has also been used to measure the rate of organelle movement in a cell. In FCS the intensity fluctuations of single fluorescent molecules (e.g. GFP) that move through a small detection volume are measured. By mathematically correlating these fluctuations, information can be obtained about the translational diffusion time of the molecules (i.e. how fast do the fluorescent molecules move) [20]. *In vivo* FCS measurements of mitochondrial matrix localised yellow fluorescent protein (YFP) yielded one or two diffusion times. Further analysis revealed that mitochondria that did not move in the cell showed the single, fast diffusion time for YFP, reflecting YFP diffusion within the mitochondrial matrix, whereas moving mitochondria showed in addition a slower diffusion time owing to the movement of the organelle in which YFP was localised. Based on these data the mitochondrial velocity could be measured [21].

The use of photoactivatable and photoconvertible proteins to measure dynamic processes

To follow the fate of a given organelle in time photoactivatable fluorescent proteins are very attractive and are extensively used [22,23]. Photoactivatable proteins become fluorescent in response to activating light. Upon photoactivation only the organelle of interest can be labelled and subsequently followed in time. Photoactivatable GFP has been used to analyse mitochondrial fusion and fission events by activating a single labelled organelle after which dispersion of the fluorescence into the organelle population (labelled by a second fluorophore) was observed and the decrease in fluorescence intensity measured [22].

For this type of research photoconvertible fluorescent proteins are also very attractive. The fluorescence properties of photoconvertible proteins (e.g. the fluorescent protein Kaede) change in response to activating light. Kaede emits green light in its native form, but is photoconverted into a bright red fluorescent marker upon exposure to UV light [23•].

In vivo *pulse-chase experiments using HaloTags*

HaloTags can be used to perform pulse-chase experiments, allowing to follow the fate of proteins/organelles that are labelled during the pulse in time.

HaloTags consists of a modified haloalkene dehalogenase, which can covalently bind various synthetic ligands, including fluorescent ones. By the pulsed addition of a cell permeable fluorescent ligand, pulse-chase experiments can be performed *in vivo* allowing to follow the fate of (organellar) proteins in time [24]. A major advantage of this method is that a single protein species can be labelled with multiple colours by the addition of different coloured ligands at subsequent time points. This approach was recently used to measure peroxisome fission and half lives in cultured mammalian cells *in vivo* [25].

Measuring protein movement by fluorescence recovery after photobleaching (FRAP)

In FRAP experiments fluorescence in a small area of the cell is bleached, followed by measuring the rate of

recovery of fluorescence in the same area. For instance, to analyse whether two organelles are physically connected, diffusion of a fluorophore from one organelle to the other can be analysed.

FRAP allows not only to measure the dynamics of (membrane) proteins within an organelle and the, movement of small organelles through the cytoplasm, but also for transport of soluble proteins, for instance between nucleus and cytoplasm [26,27].

Microscopy methods to measure autophagy

A simple method to measure autophagic degradation of a GFP-labelled organelle by autophagy is by quantifying the increase of the number of cells containing GFP fluorescence inside vacuoles/lysosomes [28,29]. This approach is most reliable using pulse-chase methods (e.g. using inducible promoters or photoactivation of a fluorescent protein), where only fusion proteins produced during a given time interval are followed in time.

A tandem-fluorophore tagged marker protein has been developed to facilitate discrimination between organelles in the cytoplasm or – upon autophagy – localised inside vacuoles/lysosomes. This pH-based sensor termed Rosella [30], consists of a pH-sensitive GFP, which loses signal in the acidic environment of the vacuole, and a relative pH stable red fluorescent protein (RFP). Before degradation cytoplasmic organelles contain a red/green-combined signal (yellow fluorescence emission), whereas upon entering the vacuole green fluorescence diminishes, resulting in red emission [28].

Concluding remarks

During the past 25 years most developments in quantitative analysis of organelle abundance and dynamics have occurred in fluorescence microscopy. In this field enormous progress was accomplished by the introduction of new and improved fluorescent probes and proteins (e.g. photoactivatable proteins), better microscopes (the introduction of the confocal laser scanning microscope and automated microscopes) as well as the development of image analysis software. However, further improvements in both the hardware and software are still highly desired to make quantitative analysis of cell architecture routinely applicable in cell and systems biology.

The imaging procedures should be fast, especially when large collections of cells have to be analysed (e.g. in an RNAi screening) or when the physical conditions in the microscope (temperature, oxygen supply, nutrient supply) are not optimal for the cells. Current problems in rapid automated imaging are among others autofocusing and the supply of objective immersion media. Because of the latter problem, generally dry objectives are used, which give images of lower quality compared to immersion oil objectives.

In automated image analysis segmentation and recognition of especially small and irregularly shaped structures is still troublesome. Therefore, at present many researchers still prefer visual scoring and manual annotation, although this is slow and error prone.

Automated microscopes suitable for a large range of applications are not yet commercially available. Those that are on the market are generally not very flexible. For instance they often contain proprietary software and image formats, which have many restrictions compared to open source software that can freely be adapted to the demands of the researcher.

Although fluorescence microscopy is very popular, the relatively low resolution is a major drawback, especially when small cells or structures have to be analysed. Despite the development of very high resolution fluorescence microscopes (PALM, STED), these techniques are currently still less suitable for the analysis of large numbers of cells or for the analysis of dynamic processes in living cells.

With respect to resolution electron microscopy is still the best method to use for analyzing organelle morphology and abundance. However, compared to fluorescence microscopy images in which only specific structures are coloured, black-and-white electron microscopy pictures in which different organelles can only be distinguished based on morphological criteria are more difficult to analyse automatically.

A combination of fluorescence and electron microscopy, as in correlative microscopy, most probably will lead to the best data for quantitative studies on organelles.

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